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THE INDUCTION OF BIOCHEMICAL AND MORPHOLOGICAL MUTANTS IN THE MOSS PHYSCOMITRELLA PATENS[1]

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ABSTRACT

Physcomitrella patens is a monoecious, cleistocarpous moss which completes its life cycle under defined conditions in 7 to 8 weeks. Sexual reproduction is readily obtained by culturing gametophytes at 15 to 19 C. Mutants were induced by treatment of either spores or protonemal cells with ethyl methane sulfonate, N-methyl-N′-nitro-N-nitrosoguanidine and X-rays. Thiamine, para-aminobenzoic acid, niacin and yeast extract auxotrophs were obtained. Growth response to various supplements was studied in the auxotrophic mutants. Five yellow mutants and two morphological mutants were induced. The chlorophyll content of the yellow mutants is reduced 35–65% of wild type. The self-sterile, para-aminobenzoic acid-dependent mutant was used as the archegonial parent in crosses with a yellow mutant and a morphological mutant. The self-sterility of the para-aminobenzoic acid-requiring mutant appears to be pleiotropically related to the auxotrophic condition, since self-sterility does not segregate from nutritional dependence in progeny of crosses. On the basis of tests with heterozygous diploids obtained by aposporous regeneration of capsule cells, two mutant alleles were shown to be recessive to their respective wild-type alleles.

THE PRIMARY OBJECTIVE of this research was to obtain biochemical mutants in the moss *Physcomitrella patens*, a multicellular and photosynthetic plant. *P. patens* was employed in this investigation because it had been used in genetic studies by von Wettstein (1925) and was known to complete its life cycle in vitro in a relatively short period (H. L. K. Whitehouse, personal communication). Other characteristics which suit *P. patens* for biochemical and genetical studies are the production of many uninucleate, haploid spores and the formation of diploid gametophytes by apospory. Thus heterozygous diploid gametophytes aposporously generated from capsule cells, as has been demonstrated in this work, may be utilized to determine the dominance of an allele. Preliminary experiments show that established procedures for mutant induction and genetic analysis may be used to study biochemical mutants in this organism.

Further studies may reveal that identification of linkage groups and chromosome mapping based on parasexual methods are possible in this morphologically and physiologically complex autotroph. Once sufficient mutants are available apospory may be employed in complementation tests to determine whether mutants are alleles and to study the functional relationship of alleles. Thus *P. patens* presents a combination of favorable aspects for studies in biochemical genetics not found among fungi and bacteria.

Induced morphological mutants in mosses have been described by Barthelmess (1938, 1941, 1953); Moutschen (1954, 1955); Bopp (1957, 1961), and Kernbach (1964). The fact that Miller, Garber and Voth (1962a, b) readily obtained biochemical mutants in the liverwort *Marchantia polymorpha* indicates that bryophytes in general may be suitable material for biochemical genetics.

MATERIALS AND METHODS—The cultures of *P. patens* used in this study were derived from a single spore isolated from nature in Gransden Wood, Huntingdonshire, England, by H. L. K. Whitehouse; subcultures were made and kindly supplied.

The life cycle—This moss is monoecious and cleistocarpous and has a short seta (0.25–0.50 mm) which attaches the capsule to the gametophore. The capsule lacks an operculum, a characteristic of cleistocarpous mosses, and contains 3–4 thousand spores, the progeny of a single fertilization, as determined by viable count. The dimensions of a spore are 20 × 40 μ; spore viability of the wild-type strain is 90–95%. A spore germinates in 48–72 hr at 25–30 C, 200–300 ft-c continuous illumination and grows into a filamentous colony on either solid or

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liquid medium with a diam of 7–10 mm in 2–3 weeks. The filaments are green protonemata which bear 10–15 gametophores per colony at the end of 2–3 weeks. At the end of this period the erect gametophores are 0.5–1.0 mm long and bear 5–6 leaf-like bracts. A colony derived from an inoculum of several protonemal or gametophoric cells develops in a similar manner.

Sexual reproduction of *P. patens* in pure culture was observed by von Wettstein (1925) and Whitehouse (personal communication). However, the critical role of low temperature in the induction of the sexual cycle was not determined by these investigators.

The present studies have established that culturing gametophytes of *P. patens* on minimal medium at 15–19 C induces the formation of antheridia and archegonia and readily results in the formation of capsules on 90–95% of the gametophores in a culture. Reports (Whitehouse, personal communication; Andrews, 1918; Bryan, 1957) of the season when capsules were found on *P. patens* in nature suggested that sexual reproduction occurs during periods of relatively cool temperatures (15–20 C). Presumptive natural conditions with respect to temperature were simulated and cultures were allowed to mature at 15–19 C. Observation of cultures grown at 20–23 C and 15–19 C for 6 months established that sexual reproduction occurs only in those cultures kept at the lower temperature for 4–6 weeks. Throughout the experiments designed to determine the effect of temperature on sexual reproduction, the light intensity was maintained at 150–200 ft-c; the light was supplied by cool-white fluorescent lamps. Light-dark regimes of 12:12, 16:8 and 20:4 hr, as well as continuous illumination, were tried at 15–19 C and were shown to have no effect on sexual reproduction. Normally, 10–20 mature capsules can be harvested from a colony in an 8-week life cycle. A culture temperature of 19–21 C diminishes the yield of capsules by 80% and at 23 C no capsules form.

Sexual reproduction was routinely induced by the following procedure: colonies derived from spores or vegetative inocula were grown on minimal medium for 2–3 weeks at 25–30 C. These colonies were then moved to a culture room where the temperature was maintained at 15–19 C. Antheridia and archegonia appeared on gametophores 7–10 days after bringing the cultures to the lower temperature. Capsules containing mature spores were produced after 3–4 weeks at the lower temperature. An aseptic culture of 15–30 colonies on solid medium in a 25 × 150-mm culture tube produced 150–300 capsules (0.5–1.0 × 10^6 spores) in 8 weeks.

Culture conditions—Hutner (1953) described the minimal medium (MM) employed in this investigation. This medium was used at one-half strength and the *p*H was adjusted to 6.0 before autoclaving. Culture media were solidified with agar which had been washed several times with distilled water and acetone and then dried in air. Organic supplements were added to MM, and the optimal levels of these supplements were determined by growth tests on cultures of the wild-type strain. Optimal supplementation of amino acids occurs in the range of 50–200 μg/ml of an amino acid. Vitamins were added to MM and optimal growth response occurs in the range of 0.1 to 5.0 μg/ml, depending on the vitamin.

For experiments requiring large inocula, such as quantitative growth tests for the response of mutants to specific supplements, 1–2 g of moss were harvested from liquid cultures, blended in a Waring Blendor and the blended protonemata washed over a filter to remove material extracted from disrupted cells. Rapidly growing cells were produced in quantity by inoculating 16 liters of medium in a carboy with 0.5–1.0 g of the blended protonemata. Carbon dioxide produced by a liter of buffer (0.25 M $NaHCO_3$ + 0.05 M Na_2CO_3) was bubbled through 16 liters of culture medium at flow rates of 600–800 ml/min. The buffer was made up fresh every 48 hr.

The light and temperature conditions were: 200–300 ft-c, 25–30 C, for spore germination and diagnostic tests for auxotrophy; 400–500 ft-c, 23–25 C, for CO_2-aerated cultures of vegetative cells for dry weight measurements in growth-response experiments; 150–200 ft-c, 15–19 C, for induction of sexual maturity and completion of the life cycle. Continuous illumination by cool-white fluorescent lamps was used in all experiments.

Cytological techniques—The chromosome number of the haploid wild-type strain was determined by observation of meiotic divisions employing techniques described by Steere (1954) and Bryan (1957).

Preparation of cells for mutagenic treatment—Spores were harvested, suspended in MM, and filtered to remove debris. The spores were then concentrated by low-speed centrifugation.

Protonemal cells for mutagenic treatment were produced using the following procedures. Four-day-old protonemal colonies grown in a carboy with CO_2-aeration were harvested by filtration, blended for 30 sec in MM and the cells collected on a plankton-net (375 μ-square pores) filter. These blended cells were inoculated into a carboy containing MM and allowed to grow for 3–4 days with CO_2-aeration. Colonies of 4–5 cells thus produced were collected on a filter. These colonies (1–2 g fresh weight) were then treated with a mutagen.

Mutation induction—Spores and protonemal cells were irradiated with a G-E Maxitron 250

X-ray machine operated at 250 kv using a 1-mm aluminum filter. The dose was varied in dose-survival experiments by varying the milliamperes at settings of 7.5, 15, and 30 ma; the time of irradiation was kept constant. The 37% survival dose (80 kr) was routinely used for mutagenic treatment of spores (1×10^5 spores/ml) and protonemal cells at 89 r/sec, 250 kv, 30 ma, with a 1-mm aluminum filter and at a distance of 15 cm.

The alkylating agent (Freese, 1959), ethyl methane sulfonate (EMS), was added to a spore suspension ($0.5–1.0 \times 10^5$ spores/ml) in 0.05 M phosphate buffer, *p*H 8.0, to obtain a 3% concentration of the mutagen. The treatment was terminated by adding an equal volume of 0.4 M sodium thiosulfate to the suspension and agitating for 10 minutes. The spores were then sedimented by low-speed centrifugation and washed several times with MM. Routine mutagenic treatment consisted of a 3-hr treatment with 3% EMS to give a survival of 20–35%.

Nitrosoguanidine (NNG) (Marquardt, Zimmerman, and Schwaier, 1964) was added to a spore suspension ($0.5–1.0 \times 10^5$ spores/ml) in MM at a concentration of 25 μg/ml. For routine mutagenic treatment of spores with NNG a 30- or 40-min exposure to 25 μg/ml was given to get a survival of 15–30%. The treatment was stopped by washing the centrifugally sedimented spores with MM. The survival of untreated spores was consistently 90–95%.

Four-day-old protonemal colonies, prepared as described previously, were treated with either EMS (1%, 1 hr), NNG (100 μg/ml, 2 hr) or X-rays (80 kr) employing the procedures mentioned above for treatment of spores. The survival of protonemal cells after these mutagenic treatments was 20–30%, or approximately one surviving cell per colony.

Mutant isolation—Following the procedure developed for selecting auxotrophic mutants in *Neurospora* (Catcheside, 1954; Woodward, DeZeeuw, and Srb, 1954), efforts were made to concentrate biochemical mutants in *P. patens* by filtration of treated spores through a plankton-net filter. In the course of many experiments filters with 50-, 100-, and 200 μ-square pores were tried. X-ray-, EMS-, and NNG-treated spores were incubated in MM ($2.5–5.0 \times 10^4$ spores/ml) on a shaker at 25–28 C with continuous illumination at 200–300 ft-c. An initial filtration was performed after 60 hr of incubation with subsequent filtrations at 12-hr intervals, terminating with the last filtration at either 108 or 120 hr. The spores remaining in the filtrate were then concentrated by low-speed centrifugation and plated on either: 50 μg/ml yeast extract, 200 μg/ml vitamin-free casamino acids, 200 μg/ml DL-histidine or 200 μg/ml L-arginine. Protonemal tips, bearing 5–10 cells, were dissected from the 2-week-old colonies and transferred to plates containing minimal and supplemented media to test for possible nutritional requirements. After 2–3 weeks colonies were examined for growth on MM. Apparent morphological and chlorophyll-deficient mutants present on the diagnostic media were transferred to culture tubes for further study.

Diagnostic tests for specific nutritional requirements—The specific requirement of an auxotroph was determined by placing inocula (5–10 protonemal cells/inoculum) on solid medium or in 25–50 ml of liquid medium containing a specific supplement. The flasks containing liquid medium were put on a shaker. The inocula were incubated under continuous illumination, 200–300 ft-c, 25–30 C.

Quantitative growth tests for the response of mutants to supplements were done with CO_2-aerated media as described previously.

Chlorophyll determination—Total chlorophyll was determined by measuring the optical density at 652 mμ of 80% aqueous-acetone extracts according to the method of Arnon (1949). Chlorophyll a/b ratios were determined by the method of Comar and Zscheile (1942).

Crossing procedures—Crosses were made by co-inoculating parental mutants on solid medium in culture tubes. The inocula were grown for 3–4 weeks at 23–26 C. The cultures were then moved to a culture room where the temperature was maintained at 15–19 C. Capsules became visible after 3–4 weeks' growth at the lower temperature. Spores were sown on a supplemented medium which allowed the survival of all the classes of progeny from a cross. The segregation of nutritionally dependent colonies among progeny was determined by transferring protonemal tips from colonies grown on supplemented medium to minimal and supplemented media.

Aposporous formation of diploid gametophytes—Protonemata grew from fragments of capsule walls on solid media to give rise to aposporous diploid gametophytes. The seta was never used to obtain diploid gametophytes because some maternal cells might adhere to the foot of the seta. Capsule wall cells, streaked through soft agar to clean the fragments of spores, were carefully inspected to see that all spores had been removed before they were used as inocula for obtaining diploid gametophytes.

RESULTS—*Chromosome counts*—A chromosome number of 28 ($n = 14$) was counted in several meiotic divisions in the wild-type strain. A cell of the wild-type strain in diakinesis is illustrated in Fig. 1. There is no cytological evidence for

multivalents in meiosis. Although not very satisfactory, the mitotic divisions which have been observed in germinating spores support the conclusion that spores of the wild-type strain are uninucleate and have 14 chromosomes.

Bryan (1957) found a diploid chromosome number of 27 for *P. patens* collected in Michigan. Von Wettstein (1925) estimated a haploid chromosome number of about 16 for *P. patens* collected in Germany. The specimens used in Bryan's chromosome counts may have been aneuploids. More evidence is needed to establish the difference in chromosome number of *P. patens* collected from various locations.

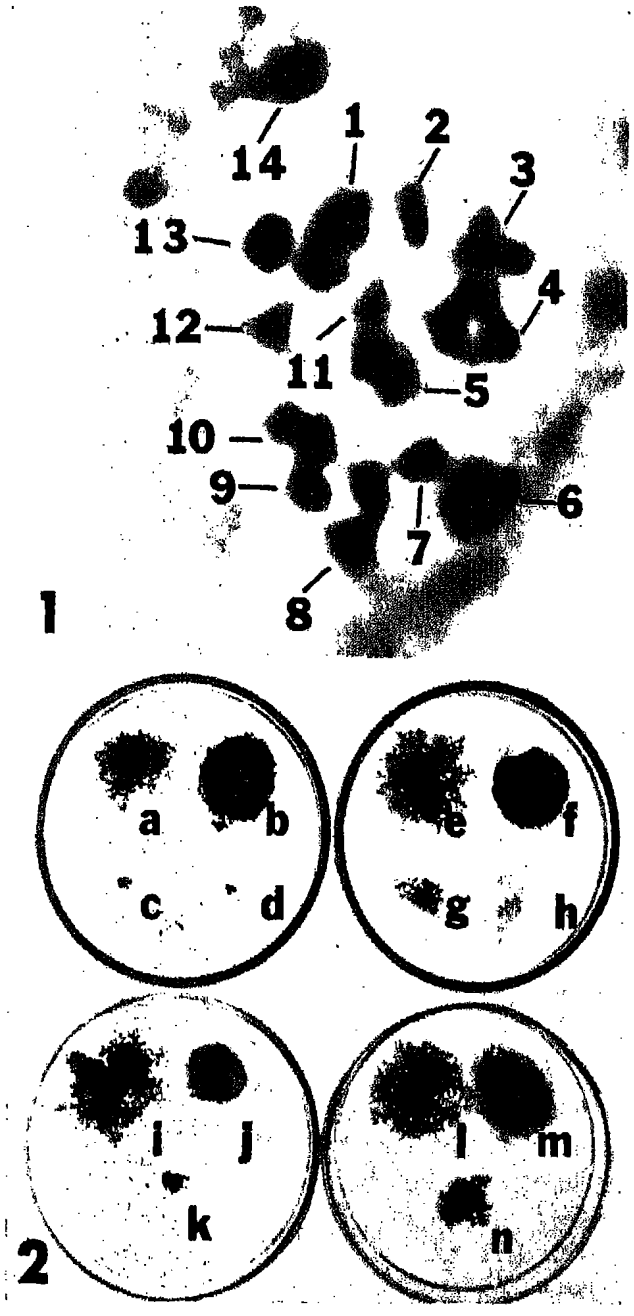

Fig. 1, 2.—Fig. 1. Diakinesis in the spore mother cell of the wild-type strain, ×2000.—Fig. 2 a-n. Phenotypes of 6-week-old progeny from the cross *gam-1* × *paba-1* (upper plates); wild-type strain, *gam-1* parent and *paba-1* parent (lower plates). Inocula for each type of colony represented were taken from the same original colony.—a-d and i-k: colonies grown on M M.—e-h and l-n: colonies grown on 0.25 μg/ml PABA-supplemented medium.—a and e: wild-type progeny.—b and f: progeny of the *gam-1* parental type.—c and g: progeny of the *paba-1* parental type.—d and h: progeny of the double mutant type.—i and l: wild-type strain.—j and m: colonies of the *gam-1* parent.—k and n: colonies of the *paba-1* parent, × 0.66.

Aposporous diploid gametophytes—Homozygous diploid strains were obtained by aposporous regeneration of capsule cells derived by selfing the wild-type strain and the morphological mutant, *gam-1*. A heterozygous diploid strain was generated by crossing *gam-1* × *paba-1*. More than 14 chromosomes were counted in the cells of the wild-type and heterozygous diploids; however, the clustering of chromosomes makes an accurate count impossible. Determination of the chromosome number and the observation of multivalents in meiotic divisions of diploid strains have not been successful as a consequence of the low frequency of capsules on diploid strains.

The data in Table 1 summarize some of the differences between haploid and diploid strains. Comparable structures in diploid strains are generally larger than in haploids.

Studies on auxotrophic mutants—A niacin-requiring mutant, a mutant auxotrophic for *p*-aminobenzoic acid (PABA), and a mutant that requires yeast extract were isolated after mutagenic treatment of spores and filtration.

The PABA auxotroph—Data on the growth response of the EMS-induced, PABA-requiring mutant (*paba-1*) to PABA and folic acid supplementation is given in Table 2. The data are based on the growth of inocula (10 mg dry weight/inoculum) incubated for 8 days in 1.5 liters of CO_2-aerated liquid media. The response to folic acid is not surprising in view of the evidence that the biological activity of PABA depends on its incorporation into the pteroic acid portion of folic acid (Welch and Nichol, 1952; Nichol, Anton, and Zakrzewski, 1955).

Niacin auxotroph—An X-ray-induced, niacin-requiring mutant (*nic-1*) grows well on either 2.0 μg/ml niacin or 0.5 μg/ml nicotinamide

TABLE 1. *Comparison of haploid and diploid strains*

Character	Haploid	Diploid
spore dimensions	20 × 40 μ	30 × 60 μ
spore viability	90–95%	5–30%
chromosome number	14	ca. 28
gametophore height	2.0–2.5 mm	2.5–3.0 mm
leaf length	1.5–2.0 mm	1.5–2.5 mm
leaf width	1.0–1.5 mm	1.0–1.5 mm
life cycle	7–8 weeks	4–6 months

TABLE 2. *Growth response of wild type and auxotrophic mutants to supplementation*

Strain	Supplement[a]	Conc. (μg/ml)	Dry weight[b] (mg)
paba-1	none		22
	p-aminobenzoic acid	0.12	132
		0.25	181
		0.50	150
		1.00	96
	folic acid	0.06	94
		0.12	51
		0.25	21
wild-type	none		248
	p-aminobenzoic acid	0.25	259
		0.50	244
		1.00	189
	folic acid	0.06	257
		0.12	221
nic-1	none		29
	niacin	0.25	37
		0.50	53
		1.00	191
		2.00	273
		3.00	232
		4.00	180
	nicotinamide	0.25	93
		0.50	177
		1.00	130
	L-Kynurenine	2.00	35
	quinolinic acid	2.00	40
	3-hydroxyanthranilic acid	1.00	34
wild-type	none		278
	niacin	0.25	262
		2.00	323
		3.00	275
	nicotinamide	0.25	280
		1.00	248
yet-1	none		20
	yeast extract	50.0	39
		100.0	106
		200.0	147
wild-type	none		288
	yeast extract	50.0	302
		100.0	233
		200.0	196

[a] Added to MM.

[b] After 8 days' incubation in 1.5 liters CO_2-aerated media; data are based on a single culture.

(Table 2). Efforts were made to determine the mutational block in *nic-1* by studying growth responses to intermediates in niacin synthesis. L-Kynurenine, quinolinic acid, and 3-hydroxyanthranilic acid have been identified as intermediates in niacin synthesis in *Neurospora* (Beadle, Mitchell, and Nyc, 1947; Bonner and Yanofsky, 1949; Partridge, Bonner, and Yanofsky, 1952). These three compounds were tested for their ability to stimulate growth in *nic-1*. Growth studies were done with concentrations ranging from 0.25–50.0 μg/ml. L-Kynurenine, quinolinic acid and 3-hydroxyanthranilic acid do not support growth of *nic-1* (Table 2). Apparently *nic-1* is sterile, as efforts over a 2-year period to obtain archegonia and antheridia have failed.

Yeast extract-requiring mutant—An X-ray-induced mutant (*yet-1*) was obtained that grows well on yeast extract at concentrations of 100–200 μg/ml (Table 2).

Efforts to find a specific supplement or combinations of nutrients that will sustain growth have failed. Archegonia and antheridia fail to develop on *yet-1* even when provided with an optimal concentration of yeast extract.

Thiamine auxotroph—A thiamine-requiring mutant (*thia-1*) was obtained from EMS-treated protonemal cells. Inocula from *thia-1* and wild type were incubated in 50 ml liquid media containing MM and MM plus 0.4 μg/ml thiamine-HCl for 10 days on a shaker. The dry weight of the inocula was 5 mg/inoculum. After 10 days' growth the dry weight of *thia-1* on MM was 6.0 mg and 31.0 mg on MM plus thiamine. The final dry weight of wild type on MM was 32.5 mg and 33.0 mg on MM plus thiamine. Neither uridine (5–25 μg/ml) nor 2-amino thiazole (0.2–8.0 μg/ml) stimulate growth of *thia-1*.

Studies on chlorophyll-deficient mutants—Chlorophyll-deficient mutants *ylo-1* and *ylo-2* were obtained from EMS-treated spores. The mutants *ylo-3*, *ylo-4* and *ylo-5* were induced by NNG treatment of protonemal cells. The total chlorophyll content and chlorophyll a/b ratios of these mutants are presented in Table 3. The data are based on cultures of protonemal inocula grown for 12 days in 100 ml liquid MM on a shaker. Since the data in Table 3 are based on single experiments, further studies are needed to determine whether the variation in chlorophyll a/b ratios is significant.

Studies on morphological mutants—Two NNG-induced morphological mutants affecting gametophore formation have been obtained. One (*gam-1*) of the two mutants was induced by

TABLE 3. *Chlorophyll content and chlorophyll a/b ratios of wild type and chlorophyll-deficient mutants*[a]

Strain	Total chlorophyll (μg/mg dry wt)	Chlorophyll content (% of wild type)	Ratio chlorophyll a/b
wild type	11.35	100.0	1.53
ylo-1	6.35	56.0	1.60
ylo-2	7.80	68.5	1.90
ylo-3	7.40	65.0	1.53
ylo-4	6.20	54.5	1.78
ylo-5	5.30	46.7	1.98

[a] Data are based on a single culture.

TABLE 4. *Bud formation on 3-week-old wild-type, gam-1 and gam-2 colonies grown on MM*

Strain	Number of buds[a] per colony
wild type	22
gam-1	0
gam-2	0.25

[a] Average of 10 colonies.

treatment of spores; the other mutant (*gam-2*) was induced by treatment of protonemal cells. The most marked difference between *gam-1* and wild type is the retarded formation of gametophores on *gam-1* colonies (Table 4). Gametophore initials appear on *gam-1* colonies 14–18 days after a spore or vegetative inoculum is sown, whereas in wild-type colonies buds form within 8–10 days. The *gam-2* mutant is phenotypically similar to *gam-1* (Table 4) but shows even more marked reduction in the number of gametophores formed. Several supplements (adenine, 50 μg/ml; glutamate, 100 μg/ml; kinetin, 1×10^{-6} M; 6-benzylaminopurine, 1×10^{-6} M) restore budding to wild-type levels in *gam-1;* the same additives restore budding in *gam-2* to about one-fourth of the wild type.

Crossing studies with mutants—Aborted sporogenesis occurs in capsules formed on *paba-1* by selfing, since such capsules never contain spores. However, capsules containing viable spores develop on gametophores of *paba-1* in crosses. This self-sterility of *paba-1* has permitted its use as the archegonial parent in crosses with *ylo-1* and *gam-1*.

The ylo-1 × paba-1 cross—The distribution of progeny among the four segregant classes was determined visually after several weeks of growth on solid minimal and PABA-supplemented media. However, the visual characterization was corroborated by growing selected individuals on liquid media for a critical test of growth response and chlorophyll content. The data presented in Table 5 are based on cultures grown for 10 days in 100 ml of liquid media on a shaker and provide evidence that visual determination of progeny phenotypes is reliable. Each inoculum had a dry weight of 5 mg. The chlorophyll determinations were done on cultures grown in 0.25 μg/ml PABA-supplemented media. The data for the distribution of progeny in crosses of *ylo-1 × paba-1* are given in Table 6. The observed deviation from the expected 1:1:1:1 ratio for unlinked genes is significant. However, the predominance of wild-type segregants suggests random segregation of the *paba-1* and *ylo-1* genes rather than linkage. The departure from a 1:1:1:1 segregation may result from the lower viability of the yellow progeny relative to wild type. Spores produced by selfing *ylo-1* normally have a viability of 70–80%.

TABLE 5. *Growth response to minimal and 0.25 μg/ml PABA-supplemented media and the chlorophyll content of progeny from a cross of ylo-1 × paba-1*

Phenotype	Media[a]		
	Minimal dry weight (mg)	PABA-supplemented dry weight (mg)	Chlorophyll per mg dry weight (μg)
yellow, PABA-dependent	6.3	18.2	3.8
yellow, PABA-independent	20.8	22.7	4.3
green, PABA-dependent	8.6	34.2	9.2
green, PABA-independent	70.2	63.1	10.1
ylo-1 parent	34.1	37.2	5.6
paba-1 parent	7.8	36.9	9.4
wild type	80.1	76.3	12.6

[a] After 10 days in 100 ml medium with shaking.

TABLE 6. *Distribution of progeny in crosses of ylo-1 × paba-1*[a]

Wild type	Parental *paba-1* type	Parental *ylo-1* type	Double mutant type
415	301	211	210

$X^2 = 99.5$; $N' = 3$; $P = 0.01$

[a] Data pooled from nine crosses.

The gam-1 × paba-1 cross—The four types of progeny, as is evident in Fig. 2, can be scored with confidence. The colonies illustrated in Fig. 2 were 6 weeks old. The distribution of progeny among the segregant classes in the cross of *gam-1 × paba-1* is given in Table 7. Assuming a 1:1:1:1 segregation, the number of progeny among the segregant classes fits the expected number quite well. The genes determining the *gam-1* and *paba-1* phenotypes are probably unlinked.

Inheritance of self-sterility in paba-1—Progeny from crosses of *ylo-1 × paba-1* and *gam-1 × paba-1* were studied to determine the distri-

TABLE 7. *Distribution of progeny in crosses of gam-1 × paba-1*[a]

Wild type	Parental *gam-1* type	Parental *paba-1* type	Double mutant type
28	34	24	38

$X^2 = 3.74$; $N' = 3$; $P = 0.2$–0.3

[a] Data pooled from three crosses.

bution of self-sterile individuals among segregant classes. A culture was considered self-sterile if it failed to produce capsules by selfing after a period of 4 months, since 93–100% of the wild-type cultures reproduce by selfing in 2 months. The data relative to self-fertility of progeny from crosses of *ylo-1* × *paba-1* and *gam-1* × *paba-1* are presented in Table 8. Self-sterility fails to segregate from the PABA-requiring phenotype, suggesting a pleiotropic relation of self-sterility and PABA dependence. Since capsules which never contain spores develop on *paba-1* by selfing, it seems likely the PABA dependence results in aborted sporogenesis rather than failure in maturation of antherozoids.

Phenotypes of heterozygotes—Experiments to determine whether the mutational changes in *gam-1* and *paba-1* are recessive to their respective wild-type alleles were done using diploids. Fragments of capsules from the cross *gam-1* × *paba-1* were put on minimal and PABA-supplemented media to obtain heterozygous (*gam-1* +/+ *paba-1*) diploids. Homozygous diploids generated by selfing *gam-1* and wild-type strains, and haploids of these strains, were used as controls in these experiments. Although unequivocal confirmation of the expected 28 chromosomes in the diploids could not be obtained as a consequence of tightly grouped mitotic figures, the number of chromosomes was clearly in excess of 14. Cultures (5 mg dry weight/inoculum) of the heterozygote, *gam-1* +/+ *paba-1*, and the wild-type diploid were incubated for 9 days in 1.5 liters of CO_2-aerated, minimal and PABA-supplemented (0.25 μg/ml) media. The dry weight of the *gam-1* +/+ *paba-1* diploid on MM was 36.1 mg and 42.0 mg on PABA-supplemented medium; the dry weight of the wild-type diploid was 35.9 mg on MM and 40.1 mg on PABA-supplemented medium. These results indicate that the mutational change in *paba-1* is recessive to its wild-type allele. Table 9 summarizes the results of experiments relative to gametophore formation on diploid and haploids. Three inocula (5–10 cells/inoculum) of each diploid and haploid were grown on agar minimal and PABA-supplemented media for 3 weeks. The average number of buds per colony was then determined. The data in Table 9 indicate that the mutational change in *gam-1* is recessive to its wild-type allele.

TABLE 8. *Segregation of self-sterility among progeny of crosses*

Cross	Progeny	No. of progeny scored	% fertile progeny
ylo-1	wild type	194	95.1
× *paba-1*	*paba-1* parental type	58	0
	ylo-1 parental type	48	93.8
	double mutant type	14	0
gam-1	wild type	28	96.4
× *paba-1*	*paba-1* parental type	24	0
	gam-1 parental type	34	97.1
	double mutant type	38	0

TABLE 9. *Comparison of gametophore formation on heterozygous and homozygous diploid and haploid strains*

Strain	Genotype	Number of buds per colony[a]	
		Minimal medium	PABA-supplemented medium[b]
diploid	*gam-1* +/+ *paba-1*	10	12
	gam-1/*gam-1*	2	1
	+/+	16	14
haploid	+	16	11
	gam-1	3	2
	paba-1	0	6

[a] Three colonies were scored in each experiment.
[b] 0.25 μg/ml.

DISCUSSION—Approximately 1,300 X-ray-treated, 850 EMS-treated and 450 NNG-treated spores selected on yeast extract medium were tested for auxotrophy. Strains requiring niacin, PABA and yeast extract were obtained. One-hundred and ninety treated protonemal cells were tested for nutritional requirements after growth on yeast extract. A thiamine auxotroph was recovered in these experiments. Approximately 5,000 colonies derived from mutagen-treated spores were selected on vitamin-free casamino acids and tested for auxotrophy. These experiments failed to provide auxotrophic strains. Five chlorophyll-deficient and two morphological mutants were also obtained in the course of this research.

Although some amino acid auxotrophs have been reported in *Marchantia polymorpha* (Miller et al., 1962a, b), *Spirodela polyrhiza* (Smith and Castle, 1960), *Ginkgo biloba* (Tulecke, 1960) and *Chlamydomonas* (Levine and Ebersold, 1960), it appears that mutations to vitamin dependence either occur more frequently or are recovered more readily in green plants, as suggested by the evidence from *Arabidopsis* (Rédei, 1965), *Chlamydomonas* (Levine and Ebersold, 1960) and this study. A satisfactory explanation for these results is lacking.

That genetic analysis is possible in *P. patens* was demonstrated by crosses involving *paba-1*, *ylo-1* and *gam-1*. The studies of inheritance suggest these three mutants are the result of mutation at three different unlinked chromosomal loci. Fortunately the self-sterility of *paba-1* has permitted controlled crosses; however, the

failure to transfer self-sterility to other mutants is a disadvantage for crosses not involving *paba-1*. A pleiotropic relation of self-sterility and PABA dependence is suggested by this failure to obtain segregation from the *paba-1* phenotype. Although the precise defect in development which leads to self-sterility has not been determined, it appears that self-sterility must be a result of aborted sporogenesis rather than a failure to produce mature antherozoids since capsules lacking spores appear on *paba-1* by apparent selfing. Apogamous development of such capsules has not been ruled out; however, this seems to be an unlikely explanation because apogamy is generally associated with aposporously generated diploid strains (Bauer, 1956, 1961; Lal, 1961; Lazarenko, 1961).

It was shown in this investigation that heterozygous diploid gametophytes can be employed to determine the dominant or recessive relation of a mutant to its wild-type allele. The generation of heterozygous diploid gametophytes indicates that *P. patens* is suited for complementation studies. Whether the yellow mutants are allelic is a problem that requires investigation. The fact that the mutant phenotypes of the morphological mutants *gam-1* and *gam-2* may be altered in the direction of the wild-type phenotype by adding supplements to the culture medium may provide a convenient experimental system for morphogenetic studies. Further studies are needed to determine whether mutagenic treatment of spores or protonemal cells is the more efficient method for recovery of mutants.

The relatively short life cycle, the ability to do crosses and the ease in culturing *P. patens* on a microbiological scale suggest that this organism may be suitable experimental material for biochemical, genetical and developmental studies.

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